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"It is characteristic of Science and Progress that they continually open new fields to our vision."—Pasteur.

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# EFFECT OF TEMPERATURE ON BACTERIAL NUMBERS IN DIGESTING SEWAGE SLUDGE[1]

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In recent years attention has been called to the influence of temperature on sewage sludge digestion. It has been pointed out that in the winter months the temperature prevailing in the digestion tanks in northern climates is too low for efficient digestion and that great benefits are to be derived from heating the tanks. A temperature of 21°C. (70°F.) has been found to be the most economical and practical limit; however, higher temperatures accelerate the rate of digestion considerably. The optimum temperature lies between 27° and 30°C.; temperatures beyond this have a retarding effect.

Little has been known about the bacteriological changes underlying the differences in the rate of digestion at different temperatures, although we know that activities of the organisms are accelerated with increased temperatures (within limits). The total numbers may or may not change. Presumably, certain groups of bacteria are stimulated but their presence in the total plate counts may be over-shadowed.

The following paper is an attempt to point out some of the bacteriological differences occurring during decomposition of solids at different temperatures.

## METHODS

The fresh solids were collected from the Plainfield sewage disposal plant and brought to the laboratory. They were divided

[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Sewage Disposal.

[2] Research Bacteriologist.

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into portions, placed into different bottles and incubated at 10°, 18°, 24°, 29.5° and 35°C. A duplicate series was incubated at the same temperatures with lime added to correct the reaction. This report deals only with the bacteriological results obtained from the experiment, which included in addition a complete series of chemical and protozoological studies.

The bacteriological analyses consisted of total plate counts and estimates of certain general groups of bacteria such as

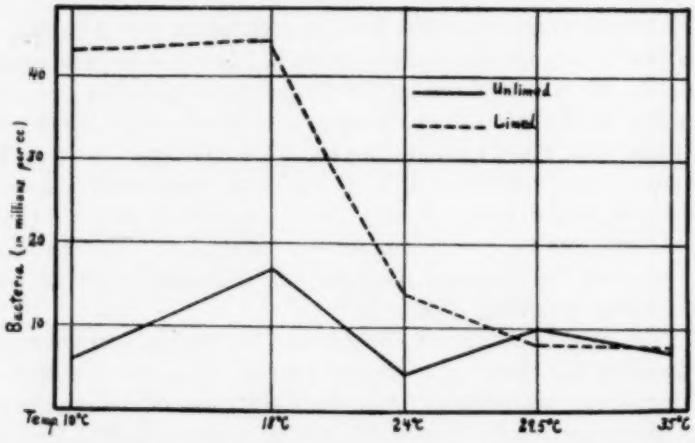


FIG. 1. AVERAGE TOTAL COUNTS OF BACTERIA IN LIMED AND UNLIMED FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

albumen digesters, hydrogen sulfide producers, nitrate reducers and lactose fermenters. The composition of the media and the procedure involved are described elsewhere in detail.[4] The analyses were made, at first, twice a week, and, after two weeks, only once a week, for a period of ten weeks.

## TOTAL PLATE COUNTS

The trend of the bacterial numbers in the unlimed materials incubated at different temperatures followed the same course

[4] Hotchkiss, Margaret. Bacteriological studies on the Imhoff tanks and sprinkling filter bed. Bul. 390, N. J. Agr. Exp. Sta. p. 49–68, 1923.

observed before in the digestion of solids, namely, a very sudden drop to a more or less uniform level which gradually rises again as digestion becomes complete. The average numbers are represented in figure 1. It can be seen that both the limed and unlimed materials at 24°, 29.5° and 35°C. had lower average numbers than at the two lower temperatures. The same kind

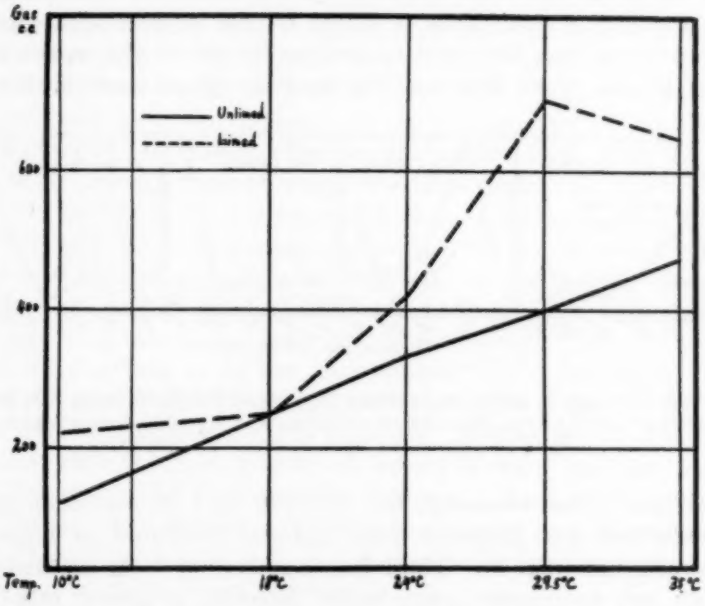


FIG. 2. TOTAL GAS PRODUCTION FROM FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES (108 DAYS)

of relationship holds when the numbers are computed on the basis of organic matter present in the sludge. When lime was added, the initial reduction in the numbers of organisms did not take place at 10° and 18°C. Lime, thus, retarded the initial reduction of the organisms which ordinarily find the medium at this stage unfavorable. Lime at higher temperatures however, did not prevent the reduction of the organisms.

The initial reduction of the numbers of bacteria might be due to the effect of high acidity produced in the first few days of digestion causing the pH value to drop from 6.4 to 5.2. The intestinal organisms in the fresh solids finding the medium unfavorable for their growth, are under these conditions greatly reduced in numbers. Low temperatures tend to prolong the life of the organisms and retard the rate of activities. The small amounts of organic acids produced at low temperatures when neutralized with lime tend to prolong the life of the organisms to a greater extent than would be the case without neutralization.

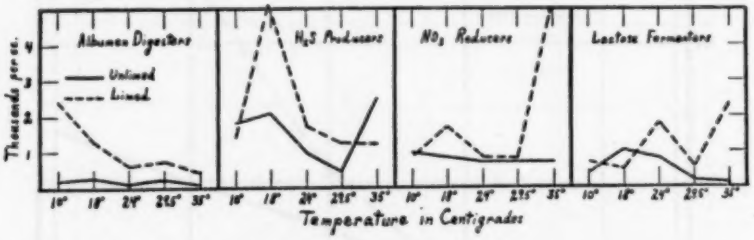


FIG. 3. AVERAGE NUMBERS OF ALBUMEN DIGESTERS, $H_2S$ PRODUCERS, $NO_3$ REDUCERS AND LACTOSE FERMENTERS IN LIMED AND UNLIMES FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

When the test was completed, after 108 days, the maximum gas production was obtained from material incubated at higher temperatures (fig. 2). Thus, high numbers of total organisms does not necessarily mean better digestion or greater rate of activity. Certain active groups of organisms, the numbers of which are over-shadowed by the presence of forms less active, must be responsible for the greater rate of digestion at high temperatures and for the greater volume of gas production. It is evident that the total plate counts do not serve as an index of digestion under all conditions.

## PHYSIOLOGICAL GROUPS

The materials incubated at different temperatures showed large and erratic fluctuations in the numbers of the different physiological groups, which could not be correlated with the

actual processes of digestion. Therefore the numbers of these organisms were averaged and are represented in figure 3.

It will be observed that the average numbers of the different groups of organisms was not materially affected by the different temperatures of incubation with the exception of the numbers of hydrogen sulfide producers which were depressed at temperatures of 24° and 29.5°C. The numbers of the different groups of organisms, then, cannot be taken as an index of digestion, since the materials at the higher temperatures, digested much more rapidly than those at the lower temperatures. Lime on the other hand has a decided influence on the average numbers of these organisms. With only a few exceptions the average numbers of the different groups of organisms were higher for the limed material than for the corresponding unlimed material. However, even with the limed material the numbers of the different groups cannot be used as an index of digestion. In the case of albumen digesters and hydrogen sulfide producers the numbers in the material incubated at lower temperatures were higher than in those digesting at higher temperatures. In other words, the numbers of these different groups do not give a true picture of either the type or the rate of digestion. It may be further noted that whereas odors with limed materials were less than with unlimed, the $H_2S$ producers were higher in all the limed materials. It would seem then that the organisms classed as $H_2S$ producers by this physiological test are not responsible for the production of $H_2S$ in the course of digestion.

The ability to digest albumen, to produce hydrogen sulfide from peptone, to reduce nitrates and to ferment lactose is not specific, *e.g.*, is not a function of one or more species. Many organisms of widely different groups are capable of bringing about the above named changes. Further, the fact that these changes are observed in the culture medium is not an indication that the same organisms are bringing about similar changes in the sewage, since environmental conditions as well as food supply—two fundamental conditions determining the activities of micro-organisms—are widely different in a culture medium and in sewage.

### ORGANISMS FERMENTING LACTOSE

Since the grouping of organisms into rough physiological groups as indicated above did not give a true index of the course of digestion and the organisms responsible for it, it became necessary to study these groups in greater detail and try to split them up into smaller groupings. For a start the lactose fermenting organisms were selected. It was realized that organisms of different physiological and taxonomic groups were included in the test for lactose fermentation, *e.g.*, intestinal organisms of the *B. coli* group, and anaerobic spore forming types.

After the test for gas formation in the lactose tubes was completed the tubes were incubated for an additional week in order to induce the sporulation of the spore-forming organisms. The tubes were then heated to 80°C. for fifteen minutes and 1 cc. of the contents transferred into fresh lactose tubes. It was assumed that within nine days most of the spore-forming organisms originally present in the sample in an inactive state would sporulate. Since the main question was to determine the numbers of the spore-forming organisms in an active and not in the spore stage, it was essential to employ an indirect method to induce the organisms to sporulate. The mere presence of the spores of the organisms in the material would not attribute to them any essential rôle.

The results obtained by the above procedure for the latter part of the digestion period are represented in figure 4. The curves show that, at the lower temperatures, a wide divergence existed between the numbers of total lactose formers and of spore-forming lactose fermenters. As the temperature increased, this divergence became smaller, so that in the two or three higher temperatures employed in the experiment the numbers of spore-forming lactose fermenting organisms constituted the majority of the lactose fermenting organisms. It would seem thus that we have here a first indication of a correlation between a certain group of organisms and digestion. It is quite probable that these spore-forming organisms belong to the genus Clostridium, the members of which group are known to be active proteolytic and carbohy-

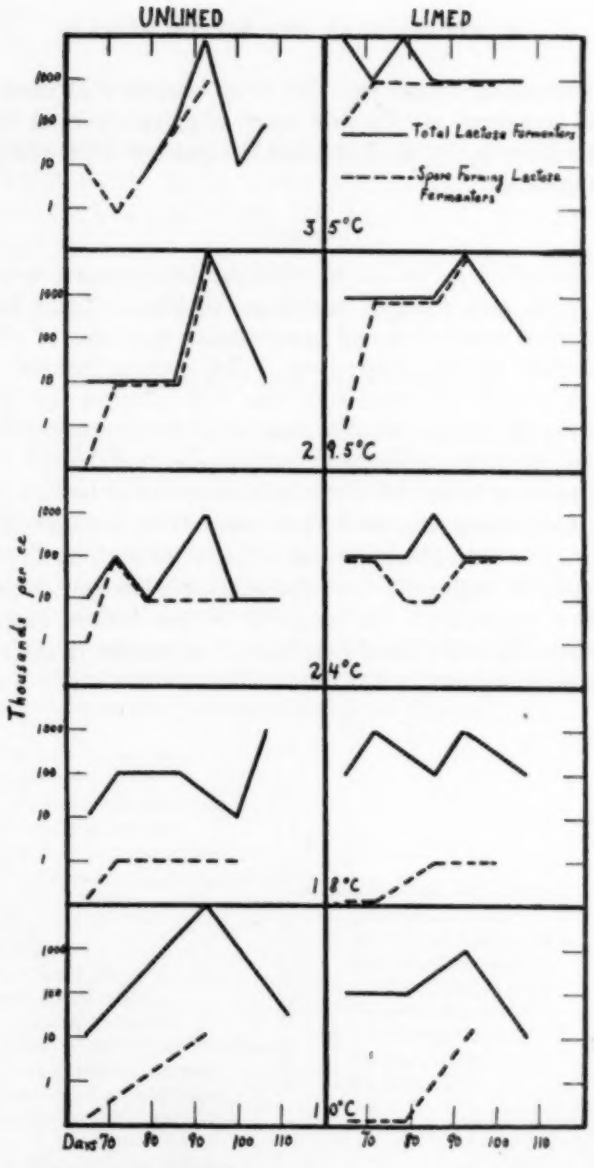


FIG. 4. COMPARISON OF TOTAL LACTOSE FERMENTING ORGANISMS AND SPORE FORMING LACTOSE FERMENTING ORGANISMS IN LIMED AND UNLIMED FRESH SOLIDS DIGESTING AT DIFFERENT TEMPERATURES FOR 108 DAYS

drate fermenting organisms. No claim is made that these spore-forming organisms are the sole cause of gassification or the only groups active during the last stage of digestion when gas production is greatest.

SUMMARY

Sewage solids subjected to different temperatures were incubated with and without additions of lime. Total bacterial counts were made and the approximate numbers of different physiological groups determined. The average number of the different groups of organisms was not affected by different temperatures, except in the case of $H_2S$ producers, but the addition of lime influenced the average numbers. A wide divergence was found between total numbers of lactose formers and spore-forming lactose fermenters. With increase in temperature, the divergence became smaller so that, at the higher temperatures employed, the spore-forming lactose fermenting organisms constituted the majority of the lactose fermenting organisms, while the total numbers of organisms (plate counts) were greatly reduced.

# A REVIEW OF THE DEVELOPMENT AND APPLICATION OF PHYSICAL AND CHEMICAL PRINCIPLES IN THE CULTIVATION OF OBLIGATELY ANAEROBIC BACTERIA

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The older methods of cultivating obligate anaerobes have been reviewed by Roux (1887), Novy (1893), Hunziker (1902), Matzuschita (1902) and Von Oettingen (1903). The work of Hunziker was especially valuable in its time but since then many important improvements in technic have been made.

The numerous methods that have been suggested for cultivating the obligately anaerobic bacteria involve, as a rule, a combination of one or more of the principles listed below under (1) reduction of oxygen tension, with one or more of those listed under (2) maintenance of reduced oxygen tension.

1. Reduction of oxygen tension
   - A. Biological reduction
     - Aerobe-anaerobic symbiosis
       - Symbiont in medium
       - Symbiont in air chamber
     - Use of animal and plant tissues
   - B. Physical reduction
     - Boiling
     - Evacuation
     - Use of inert gases
   - C. Chemical reduction
     - Agent in air chamber
       - Catalytic ignition of hydrogen and residual oxygen
       - Reduction by phosphorus
       - Reduction by iron compounds
       - Reduction by alkaline-pyro-gallol
     - Agent in medium

2. Maintenance of reduced oxygen tension
   - A. Portion of medium sealed off
     - Deep medium seals
     - Insoluble liquid seals
     - Mechanical seals
   - B. Air chamber sealed
     - Fusion of glass outlet
     - Mechanical seals

Since I have already presented the subject of biological reduction of oxygen tension in the cultivation of obligately anaerobic bacteria, with the various interesting practical applications and theoretical interpretations based thereon in "The Newer Knowledge of Bacteriology and Immunology,"[1] I shall restrict myself here to a discussion of the physical and chemical principles involved in the production and maintenance of anaerobiosis.

## PHYSICAL REDUCTION OF OXYGEN TENSION—BOILING

Boiling is the simplest means of driving absorbed oxygen out of a culture medium. It was used by Pasteur (1861) and has been used by many investigators since. All that is necessary is to place the tube or flask containing the medium in a boiling water bath for a few minutes; the reduced solubility of gases at the temperature of boiling water and the fact that each escaping bubble of water vapor behaves as a vacuum toward any dissolved gas, explain the removal of oxygen by this means. Boiling may be used to drive oxygen out of semisolid, liquefiable solid, and liquid media in tubes or flasks and may be used in combination with other methods of reduction and various methods of sealing. Generally speaking, the medium should be cooled to about 45°C. before inoculating, though this is unnecessary for the sporulating anaerobes. Boiling is unsuitable as a means of oxygen tension reduction for surface methods of cultivation.

## DEEP MEDIUM SEALS

For deep, semisolid or liquefiable solid media, boiling is all that is necessary to insure anaerobiosis, since reabsorption of oxygen generally fails to inhibit growth below a depth of 1 to 2 cm. This is the case with the deep agar and gelatin of Hesse (1885), Liborius (1886), Veillon and Zuber (1898), the deep brain medium of Von Hibler (1899), the cooked meat medium of Holman (1919), and the semisolid agar of Lignierès (1919) and others.

Deep glucose agar has been extensively used as a means of

[1] Edited by Dr. E. O. Jordan and Dr. I. S. Falk and published by the University of Chicago Press, 1928.

isolation of pure cultures. To get at the colonies in the depths it has been the practice to secure them by one of the following methods: (*a*) aspiration into a capillary pipette, (*b*) cracking the tube and its agar plug to expose the selected colony, (*c*) using cylinders plugged at the lower end with a rubber stopper and pushing the agar plug out into a sterile dish for inspection, as advised by Burri (1902), or (*d*) expelling from the cotton plugged end of an ordinary tube by heating the base so that expanding steam forces the plug out as advised by Konrich (1914). All are practical means of isolation, in the case of well separated colonies, except the last, providing aerobic organisms have been eliminated first.

One of the obstacles in the use of deep glucose agar for isolation is fragmentation by gas. Veillon and Mazé (1910) claimed this to be due mostly to hydrogen in view of the rapid diffusion of carbon dioxide and proposed the addition of 0.1 per cent $KNO_3$ for the fixation of the hydrogen. The equation supposed to represent the reaction fails to balance as given and my own effort to confirm the value of $KNO_3$ as a means of preventing cleavage of glucose agar failed for pure cultures of *B. Welchii*, *B. botulinus*, and *B. sporogenes*, though a satisfactory result was obtained with *Bacillus septicus*. But a better method of avoiding excessive gas formation is to reduce the fermentable carbohydrate content of the medium to a minimum, say 0.1 per cent, by using meat infusion 1 per cent agar without added sugar.

It is well to melt the agar surface, above the colony to be picked, with a hot spatula to avoid organisms not belonging to that colony. Theoretically the temperature attained upon the melted surface cannot exceed the boiling point, which is known to be insufficient to kill many spores, but practically there is rarely any contamination of cultures with this technic. The selected colony can best be removed by means of a heavy platinum wire, flattened at the end and bent into a tiny hook. This is preferable to aspiration by means of a capillary tube, as sometimes advocated.

Esmarch (1886) adapted his roll cultures for isolation of aerobes to the isolation of anaerobes by filling the central space with a

core of sterile agar, and Marpmann (1898) suggested forcing the inoculated agar into a thin sheet by means of a small sterile test tube inside a larger one containing the medium. The paraffin seal used by the latter is quite unnecessary. The same may be said of Friel's (1917) use of a Bunsen valve to exclude air after boiling liquid media. How his complicated apparatus can be adapted to the isolation of pure colonies from roll or slope agar cultures is hard to understand.

The use of slender tubes by Vignal (1887), Van Senus (1892), and Streng (1903) to facilitate the examination and removal of isolated colonies in the depths, relies upon the same principle of exclusion by a deep medium; the incidental reinforcement of the seal by fusion of the glass in the case of the first two is only a matter of convenience and not of necessity.

Sufficiently deep layers of liquid media also frequently yield satisfactory growth, which is largely the explanation of the successful use of the Smith fermentation tube (1893) (1899) (1905) for anaerobic cultures, except, of course, where sterile tissues are added to the media.

Rosenthal (1906) showed a direct relation between the diameter of the tube and the required depth of liquid medium necessary to insure anaerobic growth, dependent probably upon the factor of more active convection in wide tubes leading to greater rapidity of oxygen absorption.

There is an element of uncertainty in relying merely upon the depth of *liquid* media to exclude oxygen, however, except where the depth is considerable and the inoculum large, as in the production of toxin in large flasks according to Anderson and Leake (1915); ordinarily, some other seal is desirable. Kovac's (1924) claim of aerobic growth of obligately anaerobic bacteria in 10 per cent peptone broth seems to me to depend upon nothing but the increased viscosity of the broth due to the high peptone content.

### INSOLUBLE LIQUID SEALS

Insoluble liquid seals have been used in the form of oil by Pasteur (1863), lanolin by Rosenthal (1902) (1903), vaseline by

Nicolle (1902) and Beattie (1906), and paraffin by Kasparec (1896) and Park (1901).

The idea of using mercury for sealing the bend in a fermentation tube for anaerobic cultures occurred to Smith (1890), but, according to a recent personal communication, was never tried by him. In our hands, controlled tests of a variety of obligate anaerobes in glucose broth so sealed in fermentation tubes uniformly failed to permit growth, owing, no doubt, to the formation of germicidal salts of mercury in the medium.

In the use of oils, greases, and waxes, the seal is sometimes placed in position before sterilization, at other times after separate sterilization and inoculation; there is really no objection to the first method, but in the case of solid or semisolid seals, the organisms must be placed in the medium previous to the setting of the seal. Valid objections to all oil seals are their messiness, the difficulty of cleaning glassware, their interference with good staining, and, above all, a serious question as to the efficiency of liquid oils in excluding oxygen.

Vernon (1907) pointed out that olive oil and codliver oil dissolve about 4.5 times as much oxygen at 37°C. as does water, and Wolf, McGill and Harris (1917) suggested that the only real value of oil in anaerobic cultures is a possible reduction of convection currents.

Mineral oils have been preferred to saponifiable oils, as being relatively indifferent chemically. With a mineral oil, the writer has shown, in comparison with the constricted tube and marble seal (Hall, 1915), that growth is delayed unless the inoculum be large. In these experiments the combination of oil in the constricted tube with a marble seal showed that such delay was not due to antiseptic properties of the oil, and I was forced to conclude that oil was inadequate as a seal. With methylene blue as a criterion of anaerobiosis, the color returns to a decolorized solution covered with several centimeters of oil almost as quickly as without oil (Hall, 1920). This was true whether the oil had a boiling point of 300°C. as in the case of white mineral oil, or of 100°C. in the case of heptane. Fermi and Bassu (1904) were also unable, by prolonged boiling, to prevent the subsequent

darkening of alkaline-pyrogallol in media under liquid paraffin. It seems that a possible greater solubility of air in oil is partly responsible for these results but in contrast with Wolf, McGill and Harris (1917) I am inclined to attribute the return of color in reduced methylene blue solution under oil to diffusion currents in the liquid oil.

The inefficacy of the usual white mineral oil, both as regards the growth of obligate anaerobes and the protection of decolorized methylene blue, was first thought to be related to its relatively high boiling point (295° to 310°C.), but comparative tests with *B. tetani* in glucose broth layered to a depth of 2 cm. with four hydrocarbons of different boiling points, namely heptane (95° to 100°C.), xylol 137° to 140°C.), white mineral oil (295° to 310°C.), and paraffin (300° to 315°C.), showed the fallacy of this idea, for no growth was secured under heptane or xylol, while mineral oil was second only to the solid paraffin.

It was easily shown by means of heptane and xylol, layered over similar media in constricted tubes with marble seals, that there was no evidence of antiseptic action for the relatively large inocula used. The methylene blue tests definitely showed the advantage of paraffin over the liquid hydrocarbons, even in one-tenth the thickness of the latter, and both these, and the cultural tests with *B. tetani* and other anaerobes, showed that 0.25 cm. of paraffin is a sufficient seal if it remains intact.

What then is the explanation of the widespread and more or less successful use of oil as a seal for anaerobic cultures? The factor of increased depth of liquid may be partly responsible, coupled with the practice of boiling out of media, use of reducing and porous substances, and large inoculations. Larson, Cantwell, and Hartzell (1919) suggested that reduction of surface tension by the layer of oil may be an important factor, but their viewpoint awaits confirmation.

In case of the solid or semisolid greases or waxes, diffusion currents are impossible in them when they are cold, and decolorized methylene blue fails to regain its color in media so protected. Beattie (1916) pointed out the value of vaseline as a rough means of gas measurement for anaerobes. Vaseline has an advantage

in that it does not separate from the container, as does paraffin, which latter fault Kasparec (1896) very ingeniously sought to remedy by tapering the neck of his flask; the accessory bulb to hold the liquefied paraffin in his method, is however impracticable and unnecessary.

Dick (1918) used paraffin as a seal in the isolation of pure cultures of anaerobes. The material under examination was streaked out upon the surface of hardened agar in a Petri dish, covered with sterile agar, and, when this was hard, the sterile melted paraffin was poured on. The method gives only submerged colonies, of course. Northrup (1919) suggested coloring the paraffin with water-insoluble wax-soluble dyes such as Sudan III or Scharlach Roth to facilitate picking out the colonies. Fine charcoal was also found useful.

I have found a mixture of equal parts of vaseline and paraffin preferable to either alone, and we have used this compound under the coined name "vaspar" for liquid media such as broth, milk and gelatin, particularly when long time observations were required, as of *B. putrificus*. Such a seal effectively prevents evaporation, as well as the re-absorption of oxygen.

### MECHANICAL SEALS

Some of the most efficacious devices for the exclusion of air from boiled media are to be found in this group.

Nencki (1879) used a constricted tube in which a glass rod was pushed through a rubber stopper to seal off the base after inoculation. This device might have been successful had he not made it too complicated by an attempt to combine the use of evacuation and chemical reduction with alkaline-pyrogallol.

A mechanical type of seal, in addition to the depth of the medium, was invented by Wright (1900) in the form of a glass and rubber tubing device placed inside the culture tube. The apparatus is positive in action and successful even if cumbersome, as shown by Tuck (1903). I have used it successfully.

Another type of mechanical seal is Durham's (1898) (1900) fermentation tube and the similar, though vastly more complicated arrangement, of Epstein (1898) for the measurement of gas

production. Neither of these lends itself to easy and certain inoculation of the anaerobic chamber, though motile bacteria are likely to find their way past such seals more readily than air, due to the active chemiotactic responses of the bacteria, which drive them away from oxygen, so that successful growth may occur even when the inoculation is made at the surface.

One of the simplest, and at the same time, most satisfactory devices using a mechanical seal, is the constricted tube described by the writer in 1915, in which a marble, porcelain biconvex disc, or round cover slip, rests upon a constriction in a tube to seal the liquid below from that above. Kendall, Cook and Ryan adapted the idea to flasks in order to study the metabolism of anaerobes (1921) and Holman (1922) used a small test tube in place of the marble seal, but the test tube seal is quite unsatisfactory as it prevents access to the anaerobic culture. I also devised a special constricted tube with marble seal for anaerobic fermentation tests (1921). It is surprising how effectively the marble seal prevents re-absorption of oxygen into a liquid culture medium from which the air has been driven by boiling. Methylene blue remains decolorized for days below the seal although under suitable conditions of alkalinity and organic content in the solution, the color returns above the seal within a few minutes. Obligately anaerobic bacteria produce growth in suitable media under the marble seal even when incredibly small inocula are introduced. The constricted tube was designed primarily for making combined aerobic (above the seal) and anaerobic (below the seal) tests of biologic products but its greatest usefulness has proven to lie in providing suitable conditions for primary cultures of pathologic materials suspected of containing anaerobes, in the preparation of small quantities of anaerobic fluid cultures for inoculation of animals, and for serologic tests, and in the differential diagnosis and identification of anaerobes by cultural tests in meat, milk, gelatin, and fermentation media. The use of a marble seal has also been adapted to the test for relative stability in sewage by Pomeroy and Stone (1928).

Another simple method involves the use of sand as a seal in the bend of the Smith fermentation tube, or even in the base of

an ordinary tube filled with broth or other liquid medium. Using white sea sand, a black pigment-like substance is deposited by some cultures, which may prove to be of differential value. It is well, if the media be not freshly sterilized, to heat for ten to fifteen minutes in the steaming Arnold sterilizer and cool immediately prior to inoculation, though satisfactory growth may often be obtained without this precaution.

It is to be assumed that sand used as a seal in fermentation tubes acts purely as a mechanical deterrent to re-absorption of oxygen; it is probable, also, that this mechanical factor is partly responsible for the results obtained when the bend is filled with tissues as in Smith's original recommendation (1890).

It is difficult to discuss the problems of cultivation of anaerobic microörganisms in the depths of media without becoming involved in the theoretical aspects of the subject. Many anaerobes, in their growth, produce gases, such as hydrogen, carbon-dioxide, and methane, and these, in their escape, remove any traces of oxygen left in the medium exactly as bubbles of water vapor remove gases in the initial boiling. For that reason the inhibitive action of oxygen is most evident in young cultures, as Burri and Kürsteiner (1908) have mentioned. For the growth of anaerobic gas-formers in deep media, therefore, it is only necessary to provide for the initiation of growth in a secluded portion of the medium; the escape of gas soon deoxidizes the whole, so that growth occurs throughout, in the case of liquid media. These facts have an interesting bearing on the so-called aerobic growth of obligate anaerobes through the addition of various porous substances; Douglas, Fleming and Colebrook (1917) have shown the value of these substances to lie principally in the provision of anaerobic interstices for the initiation of development. The successful use of spongy platinum in liquid media for the growth of *B. botulinus*, *B. oedematis*, *B. Chauvoei*, and *B. putrificus* by Pfuhl (1907) is better explained thus than upon the idea of its acting as an oxygen absorbant. All such porous substances actually provide a mechanical seal in the liquid against rapid re-absorption of oxygen. An undeniable advantage of deep media containing mechanical seals for initial cultures is that a wide range

of oxygen tensions is provided where various and unknown types of organisms may be present.

While mechanical seals, other than those furnished by semi-solid or porous constituents of media, have been relatively little used for liquid media, they have found considerable utility in the case of solid media, especially agar, for the study and, in some cases, isolation, of single colonies in the depths. These methods are eminently suitable for the first purpose. Thus Koch used thin plates of isinglass and Sanfelice (1893), Streng (1903), and Liefmann (1908) thin pieces of glass, on the surface of plate cultures for the cultivation of anaerobes. Braatz (1895) also showed the inhibitory effect of anaerobiosis so obtained upon certain obligate aerobes. The use of agar in the special plate of Streng (1903) or between the inverted halves of a Petri dish as prescribed by Fehrs and Sachs-Müke (1908) and by Krumwiede and Pratt (1913) is merely an extension of this principle, combining, in the last case, the additional use of paraffin.

Many have used containers with outlets which might be sealed. Thus Pasteur (1861) dipped the end of such a tube under mercury, or sealed a constricted portion by fusion after inoculation. Biffi (1907) and McLeod and Soga (1914) fused the outlet tubes extending through rubber stoppers used to close their containers; though the inverted vial with the liquid medium of the former and the complete filling of the container of the latter with semi-solid medium, together with the use of sterile tissues in the depths, might be considered to provide independently adequate means of anaerobiosis. Reuschel (1906) used a pair of hemostatic forceps, and Moragas and Roig (1915) a pinch cock, for compressing a rubber tube outlet attached to a partially evacuated container. The use of the latter for anaerobic blood cultures utilizes the partial vacuum not only as a means of anaerobiosis but also for aspiration from the vein, a principle already developed in the United States by Keidel (1912).

### EVACUATION

Evacuation is also one of the earliest methods, and one that may conveniently be combined with boiling to eliminate oxygen

from semisolid or liquid media *after* inoculation, without destroying the organisms, since the boiling point is lowered by the reduced air pressure. The first use of evacuation is ascribed to Pasteur (Hunziker, 1902), and it was also utilized by Nencki (1879).

Various forms of vacuum pump have been used; our knowledge of those utilized by the earlier investigators is somewhat obscure, but Roux (1887) and Schottelius (1887) used mercury aspirators, Roux (1887), Durham (1896), Klein (1898), Süpfle (1909), Penfold (1910), and Holker (1918), water aspirators, and Meyer (1905) and Dack, Starin, and Werner (1927), mechanical air pumps. It is customary to rely upon some type of manometer, either the mercury type, which is preferable, or a mechanical vacuum gauge, for measurement of the pressure. Zupinski (1898) suggested a form of container directly attachable to a mercury manometer, in which the fall of the mercury itself produced a vacuum.

The vacuum method has been a favorite one for the study of the limits of oxygen tension in which bacteria grow, as for example, by Süpfle (1909) with cholera; Rosenthal (1906) has apparently shown the possibility of changing these limits for certain anaerobes. Yet we have as yet no adequate information on the oxygen tolerance of the obligate anaerobes generally, although Dack, Starin, and Werner (1927) have shown that while oxygen pressures equivalent to 5 cm. of mercury or more inhibit the growth of *B. botulinus* and *B. sporogenes*, growth can be secured regularly at pressures of 4 cm. or less. In computing the oxygen tensions it is, of course, necessary as Penfold (1910) pointed out in his anaerobic cultivation of intestinal aerobes, to allow for the vapor tension of water.

Methods of maintenance of vacuum appear to have been restricted mainly to the sealing of the air chamber, though there is no reason to think the sealing off of a portion of the medium would not be equally efficacious, as in boiling, where surface cultures are not contemplated.

Containers for anaerobic cultures in vacuo have been of two general types, first, those in which the vessel containing the medium is itself sealed by fusion of a connecting tube as by Pas-

teur and Roux (1887) and as according to Gruber (1887), who prepared Esmarch roll cultures in this manner, or by means of a stop or pinch cock; and second, those in which a vessel of moderate size is made to contain one or more smaller tubes or plates holding the medium. When these involve tapered ground glass stoppers there is great danger of jamming and subsequent breakage. Gage and Spurr (1913) have undertaken to obviate this difficulty by replacing the ordinary glass covers of museum jars with brass covers provided with two small metal pinch-cocks. But it is not an easy matter to construct an air tight chamber, impervious to leakage, so that the vacuum method is rarely used alone. For this reason the historical development of such containers is best reserved mainly for discussion under the next heading.

### USE OF INERT GASES

Exhaustion of oxygen by the use of inert gases is also dependent upon principles similar to those involved in boiling and evacuation, in that each bubble of introduced gas carries out its quota of oxygen. The gas may be passed through the media or it may be passed over the surface. The former is more efficacious in the removal of oxygen, though obviously not applicable to the cultivation of surface colonies, which requires the latter method. But in thin layers of media, such as are generally used for this purpose, the diffusion of absorbed gases due to variations in partial pressures at the surface probably occurs quite rapidly.

Evacuation and the use of inert gases are most successful when combined, since both are applicable to media containing heat-coagulable proteins and the use of a gas obviates the difficulty of attempting to maintain a high vacuum. Moreover, the alternate application of vacuum and gas facilitates a very complete reduction of oxygen tension. Again, Pasteur was the pioneer in the use of carbon dioxide. Carbon dioxide is now known to be somewhat deleterious through the action of the weak acid formed with water, which inhibits the growth of some organisms, e.g., blackleg bacillus, according to Kitasato (1889). It is an enticing conjecture that Pasteur was led to the use of carbon

dioxide not alone by the ease with which it may be prepared, but also because in fermenting liquors, carbon dioxide is one of the products of yeast fermentation, whose constant escape tends to remove free oxygen, thus producing a condition of true anaerobiosis. Dack, Starin, and Werner (1927) found that *B. botulinus* and *B. sporogenes* grew, and the former produced strong toxin, in carbon dioxide atmospheres equivalent to 50 cm. mercury.

But, in the early development of technic, other gases ultimately supplanted carbon dioxide, notably hydrogen, whose first use was also ascribed to Pasteur in 1861, by Roux (1887) and by Hunziker (1902), although I have been unable to confirm this. Certainly Hauser in 1885 was not the first, as mentioned both by Heim (1891) and Hiss and Zinsser, nor Lackowicz and Nencki (1884) as mentioned by Jungano and Distaso (1910), for Gunning (1877) (1878) (1879) referred to Gratama, one of his students in the University of Amsterdam, as having cultivated putrefactive anaerobes in oxygen free atmospheres of nitrogen and hydrogen. Nitrogen has never been much used as a manufactured gas for inducing anaerobiosis, though anaerobes grow well in it; hydrogen, on the other hand, has been greatly exploited. Lackowicz and Nencki (1884) generated hydrogen from sulfuric acid and iron, but it is generally produced now through the action of sulfuric acid on granulated zinc in the presence of a catalyser, such as platinum chloride, in a Kipp or other generator. On account of probable impurities in the commercial materials, the gas should be passed through a series of washing bottles, containing respectively 10 per cent lead acetate to remove sulfuretted hydrogen, 10 per cent silver nitrate to remove arseniuretted hydrogen, and a mixture of 10 per cent pyrogallic acid and 10 per cent lye to remove traces of oxygen. Even so, it is difficult to obtain a thoroughly satisfactory product.

A more modern method has been suggested in the use of steel cylinders of compressed hydrogen, produced by electrolysis of water; this would seem to be ideal, but I have given it a thorough trial in an effort to secure surface colonies of various obligate anaerobes with indifferent success. The reason for this failure is unknown; it may be due to impurities.

Durham (1896) ingeniously combined the generation of hydrogen with the absorption of oxygen by sodium hydroxide and pyrogallic acid by allowing water to act upon an amalgam of sodium and mercury. He showed that the naphtha, which clings to the metallic sodium when taken out of it, and vaporized during the process, has no deleterious action on the tetanus bacillus.

The recent use of spongy platinum and palladium as catalysers to facilitate the hydrogenation of the last traces of oxygen may be mentioned here, but their discussion properly belongs under the heading of chemical reduction.

Illuminating gas was suggested as a convenient substitute for hydrogen by Würtz and Foureur (1889). Bullock (1900), Wilson (1917) and Holker (1920) have invented special chambers for Petri dish cultures, in which they claim to have obtained surface colonies of obligate anaerobes in hydrogen and coal gas. The experiments of Ferran (1898) in acclimating the tetanus bacillus to an increased oxygen pressure by successive cultivation in atmospheres of decreasing proportions of acetylene and air are interesting in this connection, as well as in relation to his claims of aerobic growth of this obligate anaerobe; but they are of doubtful value. However, Kladakis (1890) and more recently Ludwig (1918) found illuminating gas to have a disinfectant action on bacteria. No doubt much depends upon the particular kind of gas used. The convenience which the laboratory jets offer warrants a full trial in each locality. In Berkeley, California, where gas produced from crude oil is supplied, some promising results were obtained with surface cultures in plates manipulated according to Jones' (1916) method; incomplete success at first was attributed to a slight acidity and the removal of this by passing the gas through a lye solution gave very satisfactory results.

The remarks relating to seals for the vacuum method, which is so often combined with the use of an inert gas, apply with equal force in each case, only that where no attempt is made to produce a vacuum there is less danger of ground glass stoppers jamming.

Various types of containers have been used, including tubes, flasks, special plates, and containers for ordinary Petri dishes.

### FLASKS AND TUBES AS CONTAINERS

Pasteur (1861) passed carbon dioxide into liquid media in flasks, the excess gas escaping through a mercury seal. Lackowicz and Nencki (1884) did the same thing with hydrogen. Hesse (1892) passed a stream of hydrogen through mercury into an inverted tube of solid slanted medium, while Hewlett (1894) used a yeast flask provided with two tubes, one as a hydrogen inlet and the other as an outlet dipping into a dish of mercury.

Liborius (1886) used a constricted tube, into the liquid or slanted solid medium of which, hydrogen was passed by means of a small tube sealed into the side, this and the neck of the tube being finally sealed off by fusion, inoculation having been already accomplished by means of a capillary tube passing through the cotton stopper above the neck.

Fränkel (1888), eliminating the neck and side tube, provided a rubber stoppered tube of deep medium with tubes arranged like those of a Wolff bottle, which might be fused when saturation had occurred. Petri and Maaszen (1893) also used the same idea in tubes and flasks with tubulature sealed in.

Saturation of media in tubes or flasks by means of hydrogen or carbon dioxide passed through a capillary tube in the cotton stopper and sealing by fusion of a constricted portion of the container became the subject of an amusing polemic between Heim (1891) (1892) and Ogata (1892), in which Van Senus (1892) finally claimed priority for himself as the inventor in 1890.

These methods appear to have been applied only to deep cultures until Ewell (1897), in a procedure similar to Fränkel's, combining the use of a vacuum and relying upon a warm water bath to maintain fluidity of the agar till after fusing the outlet and inlet, prepared roll cultures after the manner of Esmarch for isolated surface colonies. However, Fuchs (1890) had previously cultivated an obligate anaerobe on the surface of slanted coagulated ox serum in an inverted tube filled with hydrogen and sealed immediately by means of a paraffined rubber stopper, and we have already referred to the work of Hesse (1892).

We might mention here the use of capillary tubes of hydrogen

under slight negative pressure by Pende and Viviani (1907) as a method of somewhat doubtful value in cultivating anaerobes in this gas for any purpose whatsoever.

Perhaps the most complicated development of methods for the culture of anaerobic microörganisms in tubes or flasks with hydrogen has been reached in those procedures of Noguchi (1911) for the growth of various spirochaetes, in which the use of an inert gas is combined with evacuation, a reducing agent (alkaline pyrogallol) in the chamber and a reducing agent (sterile tissue) in the medium as a means of oxygen reduction and the use of a sealed chamber, deep medium and oil as a means of maintenance. Having once shown the possibility of cultivating the *Treponema pallidum*, it was only natural that the complex procedure should be simplified, as, for example, by Baeslack (1913), who eliminated the oil, and Bronfenbrenner (1914), who eliminated the hydrogen and the closed chamber seal, but retained the oil.

### SELF CONTAINED PLATING DEVICES

Most of the containers so far mentioned failed to provide for surface cultivation; it is the special aim of plating devices to contribute to this end, with a view to the isolation of well-separated colonies and pure cultures. As a form of container intermediate between flasks and plates may be mentioned the flattened flasks of Kitasato (1889), and Roth (1893), with inlet tube for hydrogen and cotton plug sealed with paraffin at the conclusion of the operation. Roth, later, eliminated the tube as a part of the flask, finding it an unnecessary complication, for which a tube passing through the cotton stopper might be substituted just as well. Berner (1904) also invented a flattened flask with two gas tubes, one provided with a ground glass stopper, the other with a ground glass cock, a combination almost impossible to sterilize without breakage.

Special types of plates with matched perforations for the passage of gas were invented independently by Gabritschewsky (1891) and Kamen (1892); sealing was accomplished by twisting the holes out of alignment, the former providing also for the use of a chemical reducing agent, alkaline-pyrogallol.

Migula (1895) used two glass dishes, one being provided with a tube for the passage of hydrogen which was allowed to escape through the hydrocarbon oil used in the larger one for a seal. Various other forms of double plates for paraffin sealing and tubulature for gas were invented about this time by Beck (1897), Epstein (1900), and Bombici (1902); all are open to the objections of their high initial cost as unusual apparatus and their great fragility. A more promising device of this general class was that of Jones (1916), consisting of a turned iron base with inlet and outlet holes for gas and a circular trough on top, into which a streaked Petri dish might be luted by means of paraffin previous to sweeping out the oxygen by means of hydrogen, the gas tubes being sealed by fusion thereafter. The writer had indifferent success with the method of Jones in the use of hydrogen gas from a Kipp apparatus but this was considerably improved in the use of gas supplied from the laboratory taps, as already described.

The principal objection to all such devices is the difficulty of obtaining them, which rests in the final analysis upon the fact that none has proven of such outstanding superiority as to make commercial production feasible.

### JAR-LIKE CONTAINERS

Coincident with the development of these special plating devices for single cultures, there have been invented various methods whereby several plate cultures might be placed in a single container, usually a bell or jar.

Liborius (1886) combined the use of evacuation and hydrogen in a bell jar, provided with a rubber tube inlet and outlet and pinchcocks for sealing. This jar was held down on a rubber ring by means of clamps and provided for surface cultures on several agar plates at once. Variations, but not improvements, were introduced by Blücher (1890) and Botkin (1890). The former inverted a lead-weighted bell in a deep dish of glycerin, upon which the dish of medium was floated, hydrogen being introduced under pressure at the top of the bell through an inlet containing cotton, which was afterwards sealed by means of glycerin poured into the clamped end of a rubber tube; the latter used paraffin

as better than glycerin for sealing the base of the bell, introduced his gas through U tubes passing under the lips of the bell, and included a rack to hold the dishes. Botkin's bell was sterilized by means of corrosive sublimate solution and dried, first with alkali and then ether. The same general procedure was followed essentially by Hesse (1892) except for the use of a mercury seal instead of paraffin for the base of the bell.

The well known Novy jar was first described in 1893 and again in 1894. It provides a base with removable cap, containing a glass cock ground in, and carrying both inlet and outlet tubes. It has been used as a combination method for evacuation alternated with hydrogen or other gases. The stoppers of Novy jars in their present form almost invariably jam under vacuo. It is not surprising therefore that other forms of apparatus continued to be suggested, such as Hewlett's chamber for plate cultures (1894), with two tubes providing mercury valve seals, and Lubinski's jar with sealed in tubulature for oil valves (1894). Zettnow (1894) and Mereschowsky (1903) also contrived cheap metal containers to be used with hydrogen, sealed in by paraffin, in connection with alkaline-pyrogallol as a reducing agent, and Kedrowsky (1895) used a crystallizing dish on whose ground glass cover plate, at diametrically opposite points, perforations to permit the entrance and exit of gas could be turned out of alignment to perfect the seal, the ground glass surface having been smeared with vaseline. Such a plate could be made to contain the medium, as in the case of the methods of Gabritschewsky (1891) and Kamen (1892), or used with one or more enclosed Petri dishes. Durham (1896) devised a system of tubes and museum jars covered with an iron plate clamped down, and generated hydrogen by allowing a soda solution to act upon pyrogallic acid and sodium amalgam, an ingenious but clumsy method, of doubtful value. Petri (1900) advocated a plate and bell jar into which hydrogen could be passed through tubes in the rubber stopper in the top and in which alkaline-pyrogallol served both as a reducing agent and a chemical criterion of anaerobiosis. Bullock (1900) also used a bell jar with glass stopcocks sealed into the top, and Bordet (1904) adapted a desiccator to the same purposes, while

Gage and Spurr (1913) proposed an ordinary candy jar with a metal plate, perforated and provided with metal stopcocks, to avoid the breakage so often encountered with glass cocks, for passage of the gas. In 1917, Wilson devised a special base with inlet and outlet tubes to accommodate a bell jar and several Petri dishes.

Several modifications of the jar principle have been made in which there is no use of inert gases, reliance being placed on other means of oxygen tension reduction, such as the use of chemical reducing agents; these are discussed in their proper places.

The possible value of having a combination of methods, such as deep media with reducing agents, vacuum, inert gas, and chemical absorption, was exemplified in the work of Laing (1904) with several anaerobic bacteria, and more especially of Noguchi (1911), in cultivating the *Treponema pallidum*.

It seems to me that the use of inert gases is only justified for securing surface growth of the obligate anaerobes, whether for isolation or for observing the form of the colonies or their action upon certain media, since, as already pointed out, deep methods of cultivation or methods with mechanical or insoluble liquid (i.e. wax) seals are entirely adequate for purposes other than surface cultivation. The valid objections to surface cultures for isolation have been clearly stated in another place (Hall, 1920). When surface cultures are desired, it is simpler and preferable from a practical viewpoint to rely either wholly or in part upon chemical methods of reducing the oxygen tension.

## CHEMICAL REDUCTION OF OXYGEN TENSION

Chemical reduction of oxygen tension provides some of the most useful means of cultivating obligate anaerobes. Chemical reducing agents may be used in two ways, either in a closed chamber but not in contact with the cultures, or in the media themselves.

### CHEMICAL REDUCING AGENT IN CLOSED AIR CHAMBER

#### *Use of a catalyser to ignite hydrogen and residual oxygen*

During the war a method was developed by Laidlaw (1915) for cultivating obligate anaerobes upon the surface of solid media,

which combined the use of an inert gas and chemical reduction, depending upon the well known fact that spongy platinum absorbs an inflammable gas with sufficient generation of heat to ignite the gas in the presence of oxygen. The idea was first applied in tubes containing slanted media, or sometimes liquid media, provided with stoppers carrying a small mass of spongy platinum mounted upon a wire. The culture medium was inoculated in the usual manner. The cotton stopper was then removed and a small jet of hydrogen from a generator was led into the tube for a few minutes. The spongy platinum was then slightly warmed and quickly placed in the tube; if successful it would immediately glow until all of the remaining oxygen was consumed. Laidlaw also tried spongy palladium without advantage, though McIntosh and Fildes (1916) found palladium cheaper in cost, more efficacious in operation, and superior to all other methods for the surface isolation of pure cultures. The method was also used successfully by Stoddard (1918) but my own experiences with it at that time were not successful, owing to the invariable occurrence of accidents, either failure to ignite the hydrogen or violent explosions; no surface cultures could be obtained.

In 1917, Smillie devised tubes and jars into which hydrogen could be led and a platinized asbestos catalyser heated after closure. With this he claimed success, not only in cultivating the common anaerobic bacilli but also the "globoid bodies" of poliomyelitis. A similar device for use with palladinized asbestos was described in England by Fildes and McIntosh in 1921, while in America, improvements in the arrangement of the heating element were made by Brown (1921) to secure better contact of oxygen and hydrogen with the catalyser and by Brown (1922) and Richardson and Dozier (1922) to avoid explosions and to prevent cracking the glass covers by the heat. The most highly developed form of anaerobic jar is that of Wilson (1928) based upon the original ideas of Laidlaw and of Smillie, in the form of an all metal jar with provision for evacuation, inert gas, and catalytic reduction activated by electric current.

A slightly different trend manifested itself in the "central

station for catalysis" devised by Boez (1925) (1926) to which one or several containers of similar or different types might be attached for reduction of oxygen tension.

*Use of phosphorus as a reducing agent*

The earliest reference to the use of this vigorous reducing agent found by the writer is that of Gunning (1877) to the work of Gratama, already mentioned in connection with the use of hydrogen. Gratama cultivated bacteria in atmospheres considered to be oxygen-free because of the failure of phosphorus to ignite, thus using this element as a criterion of anaerobiosis rather than as a primary means of reducing oxygen pressure. Sellards (1904) appears to have been the first to utilize phosphorus for this purpose; he cultivated the tetanus bacillus in a hanging drop by this means and studied the effect of anaerobic growth upon the metabolic activities of certain aerobes, a number of which, for example, liquefy gelatin only aerobically. He found that certain precautions had to be followed in handling phosphorus, due to its poisonous and inflammable character and to a possible deleterious action of phosphoric acid upon the cultures, which could be prevented by the provision of an absorbing body of alkali. Surface cultivation of obligate anaerobes was not described by Sellards, but Bushnell (1922) succeeded with several species. There was some difficulty in the formation of deposits upon the surfaces of plates and cotton plugs and in the decolorization of red wax pencil labels, but these objections were overcome by covering the cultures with paper and using blue pencils. Bushnell found that it was unnecessary to use lye to absorb the fumes, water serving equally well. Bushnell's success was confirmed by Varney (1926) who, instead of fruit jars and fireless cookers, used glass museum jars with metal racks for the Petri dishes. Chapman (1928) found Varney's method useful in the selective isolation of *B. Welchii*.

*Use of iron compounds as reducing agents*

Drossback (1893) postulated the presence of anaerobic bacteria growing at 37°C. as the true criterion of water pollution and

proposed their detection by means of cultures in a desiccator sealed by a wax and oil mixture. From this desiccator it was proposed to absorb the oxygen by iron "oxydul" prepared by the action of strong lye on iron chloride. This work is unimportant, but the reducing action of iron and iron salts in culture media used for anaerobic organisms presents a promising field, in which important beginnings have already been made by Scott (1926).

*Use of alkaline pyrogallol as a reducing agent*

No chemical reducing agent has had a more extensive use in bacteriology or resulted in more kinds of fantastic apparatus for bacterial cultures than the mixture of strong alkali and pyrogallic acid. The vigorous avidity for oxygen possessed by such a mixture is well known, and, while the chemical textbooks are strangely silent on the question of the exact reactions involved, carbon monoxide, carbon dioxide, and acetic acid are said to be among the end products formed (Cohen, 1920). Nencki (1879) used such a mixture, perhaps unnecessarily, to reinforce the exclusion of air in his constricted tube and plunger device for liquid media; but the first significant application of this principle was by Hans Buchner (1888) who believed that there was little or no evidence that the volatile products formed interfered with bacterial growth under practical conditions. He invented the method of stoppering a small tube of inoculated liquid or solid media inside a larger one containing 1 gram pyrogallic acid crystals and 10 cc. 10 per cent lye per 165 to 175 cc. air space. In the suggestion of Esmarch roll cultures and plate cultures in a closed bell jar, Buchner essentially anticipated the principal lines of development of apparatus subsequently designed for the more efficient utilization of this principle, i.e., methods using tubes and methods using plates.

In addition, we may mention certain methods invented for the observation of bacteria microscopically under anaerobic conditions, as, for example, the hollow-ground slide method of Nikiforoff (1890), the moist chamber methods of Braatz (1890) and Itano and Neill (1921), for the cultivation of anaerobes in hanging drops, and the flattened tube device of Dunham (1897) for

the study of motility under anaerobic conditions, all using alkaline-pyrogallol as a means of reducing the oxygen tension.

### PLATING METHODS USING ALKALINE-PYROGALLOL

More important are those modifications which aim to provide conditions such that well-separated surface colonies may be secured as a step in isolation. While Buchner's procedure pertained mainly to the use of tubes, the earliest subsequent development concerned the applicability of the technic to plate cultures, which have been obtained by modification in two general directions, first, by the invention of special plates or combinations of plates to contain the absorbent along with the culture, and second, by the use of bell jars, desiccators, or similar vessels in which ordinary Petri dish cultures might be placed. Both schemes have been utilized with alkaline-pyrogallol alone as the means of reducing oxygen tension or in combination with other methods.

### SINGLE PLATING DEVICES

Blücher (1890) described a Petri dish fitted to a saucer with vaselined ground edge, to contain a mixture of lye and pyrogallic acid in the same chamber as the culture, while Gabritschewsky (1891) devised a plate with a moat for the reducing mixture and a ground glass cover with perforations for the passage of inert gas, closure being accomplished by rotating the cover on its vaseline seal, thus throwing the perforations out of coincidence. The principle objection to both plans is that the special plates are not now commercially available, an argument that applies even more to the very complicated and expensive apparatus of Trambusti (1892) and the tubulated Petri dish of Epstein (1900). Turro's (1902) use of the half of a plain Petri dish to hold the alkaline-pyrogallol mixture with a slab of agar carried on a plain glass cover supported on small blocks and luted into position by means of paraffin was a step in the direction of simplicity, but required rapid work in sealing after the mixture was made. The methods of Stüler (1907) and Zinsser (1906), seem to fail in their somewhat extravagant use of reagents and lack of ade-

quacy in the oil seal; my own attempts to cultivate the tetanus bacillus on the surface of glucose agar by the method of Zinsser resulted only in failure. Ogata and Tagenouchi (1914) advocated a similar method with the addition of a mercury seal, which is of doubtful value in the form proposed. The saturation of cardboard disks with definite amounts of pyrogallic acid for use in Petri dishes, proposed by Hammerl (1901) and commercialized by Lentz (1910) requires dishes of unusual depth; the lack of suitable dishes has prevented a fair test of this method in our hands.

Somewhat similar is the method of Saiki (1909) in which NaOH solution seeps through a pasteboard box sealed up in a system of three crystallizing dishes with liquid paraffin and glycerin so that it saturates the pyrogallic acid after closure.

Heim (1910) and Löwi (1919) placed the alkaline-pyrogallol mixture in the cover of a Petri dish sealed to the inverted bottom containing the culture by means of plastiline.

Simonds and Kendall (1912) utilized a vacuum in mixing the alkali with pyrogallol in a common flat bottle; they also secured satisfactory surface cultures by the method of Rickards in which tubes or Erlenmeyer flasks were inverted in a beaker of alkaline-pyrogallol, a wasteful practice, as contrasted with their own method and some others.

The method of McLeod (1913) had for its object the sealing of the air space containing the culture prior to making the mixture of alkali and pyrogallol, so that all the oxygen absorbed might be derived with certainty from within the chamber. This device comprised a glass plating dish with upcurled edges, to prevent escape of infected droplets, which after streaking was to be inverted over a porcelain capsule partitioned for the receipt, on one side of pyrogallic acid, on the other strong alkali, through a central perforation. The two were then luted together by means of modelling clay and the mixture was made after the seal was completed, an altogether ideal arrangement it would seem, since a large area is provided for absorption. In England, Henry (1917) successfully used essentially the same idea in an improvised assembly of sheet iron can tops and Petri dishes, modifica-

tions of which were later described by Wilson and Steer (1918); while in Germany, Brekenfeld (1924) invented a Petri dish over rubber washers on a plate and combined the use of vacuum with that of alkaline-pyrogallol.

### COMPOUND PLATING DEVICES (JARS, BELLS)

The Novy (1893) (1894) jar principle already discussed in connection with inert gases was utilized with reducing agents by Arens (1894), who recommended the isolation of *B. tetani*, *B. Chauvoei*, and *B. oedematis-maligni* from Petri dishes in a jar containing sand mixed with dry pyrogallic acid crystals, upon which a dish of 10 per cent lye could be tipped after sealing. Arens dispensed with the use of hydrogen. Zettnow (1894), on the other hand, combined the use of hydrogen with the alkaline-pyrogallol. The use of sodium mercury amalgam with pyrogallic acid and water, with incidental production of hydrogen, by Durham (1896) has been already noted. This method was conveniently operated in a candy jar with iron top and metal pet cocks for evacuation by water pump, in this respect antedating the suggestion of Gage and Spurr (1913). Ayer's (1910) also invented a less fragile type of jar in spun copper with rubber stoppers for use with gases, and alkaline-pyrogallol.

Ucke (1898) appreciating the value of making the mixture of alkali and pyrogallol after closing the apparatus, placed the pyrogallic acid in a small beaker floating in a larger beaker of alkali, running in, under partial vacuum, sufficient water to sink the smaller dish.

Klein (1898) used a simple bell jar, sealed on a plate with beef tallow and wax, for evacuation by water pump through a single rubber stopper and tube which was later sealed by means of a pinch cock, in combination with alkaline-pyrogallol.

Kabrhel (1899), in testing the anaerobiosis of media with methylene blue in an apparatus identical with Klein's, except for the use of a ground glass stopper and cock instead of rubber stopper and pinch cock, suggested leaving the covers off the culture dishes, in order to permit free diffusion of oxygen from the medium. Petri's suggestion (1900) was similar to that of

Klein, except that a doubly perforated rubber stopper in the top of the bell offered an opportunity to pass hydrogen through the air chamber as an additional means of oxygen reduction. Bullock (1900) used a bell jar with sealed-in glass tubes, having ground glass stop cocks, a more expensive, and fragile, but no more efficacious apparatus. Bullock improved the seal by using *"unguentum resinae"* and utilized a partial vacuum to draw the alkaline solution upon the pyrogallic acid crystals after the bell had been filled with gas, either hydrogen or coal gas. Bullock pointed out that, were it not for the intense heat generated by the solution of KOH in water, which might damage the cultures, water might be drawn upon a mixture of dry granulated lye and pyrogallol. Ruzika (1901), using Kabrhel's apparatus, apparently overlooked this objection, in advocating the mixture of solid KOH with a solution of pyrogallol instead of the reverse. Harrison (1902) also used evacuation as a means of emptying an inverted vial of alkali upon the pyrogallic acid crystals, a method recently rediscovered by Ehrenberg (1927).

Fremlin's simple use of a crystallizing dish with ground glass cover first (1903) for a single Petri plate, later (1904) enlarged to accommodate several, was claimed to be successful for surface colonies of *B. tetani*; methylene blue in alkaline solution of sugar or methylated spirit, used as a criterion of anaerobiosis was decolorized, but the alcoholic solution, though more delicate, was not recommended because of its volatility, since it might inhibit bacterial growth.

In view of the relatively well-developed procedures just outlined, the use of a simple bell sealed down with paraffin by Slupski (1901) in his study of the anaerobic growth of the anthrax bacillus and the use of the metal dish in which Mereschowsky (1903) combined the reducing action of hydrogen with alkaline-pyrogallol, and the cumbersome assembly of Lode (1925) seem to involve a retrogression in technic or at least a useless duplication of description. On the other hand, the application of a desiccator to the purposes of the Novy jar by Bordet (1904) and Laing (1904) would seem to be satisfactory. We have already referred to the device of Wilson (1917) as another successful combination of the

hydrogen and reducing agent principle for plating, and this was elaborated by him in 1925 into a porcelain vessel of special design to combine the principles of evacuation, inert gas, and alkaline-pyrogallol for plate cultures.

### USE OF TUBES IN THE ALKALINE-PYROGALLOL METHOD

The first work done with reducing agents, i.e., that of Gunning (1877) (1878) (1879), involved the use of tubes, some of which were quite fantastic, but the first really successful device was that of Buchner (1888). It is interesting to note that the original inclusion of a small tube of inoculated medium inside a larger tube containing the alkaline-pyrogallol by Buchner has continued to be one of the most practical methods for the surface cultivation of anaerobes up to the present time. Buchner must have failed, however, to appreciate the necessity of emphasizing the applicability of his procedure to surface cultivation, if we can judge from his illustration, which only shows a deep culture, for which, as we know, special chemical means of anaerobiosis are superflous.

Buchner's combination of tubes led to several modifications, for example, Lubinsky (1894) suggested that the inner tube be supported in a glass stoppered cylinder by means of a perforated cork, and Wright (1900) (1901) proposed that the pyrogallic acid-lye mixture be carried in a cotton plug under the rubber stopper. But, like Buchner, Wright apparently failed to appreciate the unimportance of the method for any but surface cultures, and so pictured a broth culture. Wright's method was simple and effective and required only attention to certain details of technic including the use of solid media, a rubber stopper and seal, and inversion of the tube, to place it in the front rank of many devices for surface cultivation of anaerobes. Crendiropoulo's (1910) combination of the use of hydrogen with Wright's method is superflous. Mellon (1919), who recently pointed out the defects in Wright's original method, offers an alternative procedure in covering the plug with paraffin which is then perforated, treated with alkaline-pyrogallol and capped with rubber.

Whatever the results may be, it seems to involve considerable unnecessary and messy labor.

It seems almost as if the most conspicuous effect of Buchner's simple application of the principle of absorbing oxygen by means of alkaline-pyrogallol to the cultivation of obligate anaerobes, was to stimulate many bacteriologists to invent as complicated devices for this purpose as possible. Curiously such devices are almost always described as "simple"! Thus Omelianski (1902) devised an outer cylinder capped by a glass cover set in a mixture of wax and vaseline, E.F. Smith (see Hunziker, 1902), an inverted U tube, one leg of which held the slanted culture, the other the reducing mixture (used successfully by Nichols and Schmitter (1906) as well as myself), Buchanan (1914), an inset absorption appliance consisting of a small perforated tube carried on the lower end of the rubber stopper to contain the alkaline-pyrogallol solution (not very successful in my hands), Dimond (1915), a liquid paraffin seal between the inverted inner culture tube floating in the alkaline-pyrogallol solution and the open outer tube, and Giltner (1915) and Kollath and Quast (1925), H tubes (of which the prototype was employed by Gunning and his students (1877), with phosphorus as a chemical reducing agent and criterion of anaerobiosis.

Many years ago I used Buchner's method for the isolation of *C. acnes* on slanted oleic acid agar. The tubes were 15 by 2.5 cm. and 12 by 1.5 cm. respectively. Into the larger, about 10 cc. 10 per cent commercial lye solution were pipetted, on top of which a loose plug of absorbent cotton was placed, the tube then being filled with about 1 gram, usually unweighed, of pyrogallic acid crystals. The medium having been inoculated in the small tubes, it was then used to mix the crystals with lye by pushing these and the cotton plug barrier down into the lye solution, at the same time closely stoppering with a previously well-fitted rubber stopper in the large tube. Either end of the small tube may be inserted first, but better results were secured if the cotton plug was left out and the open end itself submerged in the lye pyrogallol solution. When growth occurred, the tube could be rinsed and replugged with a clean cotton plug from a sterile

tube and handled like any aerobic culture. For the easy removal of the small tube, a vacuum plunger, made by fastening a rubber stopper onto a glass rod and melting out a concave face by means of a hot test tube, was used.

The simplest and most effective means that I have yet found, after testing many schemes and devices for the surface cultivation of obligate anaerobes, is Wright's method, modified by the use of solid slanted media of any kind, a rubber stopper seal, and inversion of the tube to prevent the absorbing solution from running upon the culture. The tubes are inoculated with anaerobic bacteria in the same manner as with aerobic bacteria; the cotton stopper is cut off with a pair of scissors and pushed into the tube about 2 cm.; the space is filled with crystals of pyrogallic acid; about 2 cc. of 10 per cent lye are poured upon them; a previously fitted rubber stopper is inserted, pushing the lye solution through the crystals into the cotton stopper, and at the same time the tube is inverted. A large area of absorption is provided by the cotton fibers and, if precaution is observed to have an excess of pyrogallic acid, few failures result.

When I first used the above method, about one-fourth of the cultures failed to grow. The reason was not understood until Rockwell (1921) (1924), whose studies have shown the apparent need of anaerobes, perhaps all bacteria, for carbon dioxide, proposed to substitute a mixture of $NaH_2PO_4$ and $NaHCO_3$ for the lye in Wright's method to provide the required $CO_2$. I was never very successful in using Rockwell's formula though it seems quite probable that the principle upon which it is based, is sound. So far as practical results in growing the obligate anaerobes are concerned, I believe that the absorption of carbon dioxide can be prevented by having always an excess of pyrogallic acid; and the greatly improved growth when this precaution is observed justifies this view.

Surface cultures upon which isolated colonies appear may be used for securing pure cultures but exclusive reliance should never be placed in isolation of obligate anaerobes upon surface cultures, because we have as yet no means for measuring the degree of oxygen tension reduction in such cultures and there is

always the possibility that organisms may be present whose particular requirements in reduced oxygen tension may not be met. Any such might, of course, be transferred along with those that have grown, so preventing purification. Deep methods of isolation are therefore preferable (Hall, 1920) but surface cultures are useful in determining hemolytic action on blood agar or liquefaction of proteins such as coagulated serum, and in studying the form of colonies and of the individual organisms.

## THE USE OF CHEMICAL REDUCING AGENTS IN CULTURE MEDIA FOR OBLIGATE ANAEROBES

### *Glucose as a reducing agent*

The most important chemical reducing agent used in media for the growth of obligate anaerobes is glucose. Liborius (1886), while not admitting the indispensability of fermentation in anaerobic growth, clearly recognized its stimulating effect. The relation of sugar to anaerobiosis has been well reviewed by Theobald Smith (1895), who recalled the view of Pasteur, Nägeli and Escherich that fermentation is an essential element in anaerobiosis. Kitasato and Weil (1890) pointed out that sugar may serve not only in reduction of oxygen, but in nutrition as well, and Beyerinck in 1893, according to Smith, concluded that some reducing material *must* be present in anaerobic growth. But the experiments of Smith himself show most clearly the value of glucose as a determining factor in the fermentation tube, where no growth of obligate anaerobes could be obtained without it and facultative anaerobes grew only in the open arm of the tube. It must be recognized now, of course, that many anaerobes can be cultivated in the absence of fermentable carbohydrates, providing that more efficacious means of excluding oxygen than those afforded by the fermentation tube be provided, and that fermentation is not an indispensable factor in anaerobiosis. Smith (1895) thoroughly appreciated the deleterious effect of a fermentable sugar in the medium upon the viability of most aerobic and anaerobic bacteria, a point of view re-emphasized by Ucke in 1898, who spoke of glucose as a two-edged sword. But its harmful effects occur only when in excess, and are indirect, that

is, they are due to the acid formed; when there is less than an excess the limiting $H^+$ ion concentration is not reached and in most cases the culture again becomes neutral or even alkaline through de-aminization of the protein. It seems clear also that for most of the obligate anaerobes, excepting those that do not ferment sugars, sporulation probably occurs only in sugar-free media, so that not only is the organism exposed in the presence of an excess of fermentable carbohydrates to the adverse circumstance of acidity, but its own usual means of defence may be weakened or lacking.

*Other chemical reducing agents*

Kitasato and Weil (1890) undertook to find a stronger reducing agent than glucose which would be effective in a sufficiently low concentration not to inhibit growth. The substances studied fell into two groups, first, those which in alkaline solutions strongly reduce or absorb oxygen, and second, those whose color depends upon the presence or absence of oxygen. Among the various organic substances tested, sodium formate was found effective as a reducing agent in as low a concentration as 0.1 per cent and non-inhibitive for *B. tetani*, *B. chauvoei*, and malignant oedema bacilli up to 3 per cent. The nearest approach to this in the first group was in the case of pyrogallol, hydrochinon, and resorcin, which, while effective at 0.1 per cent, are inhibitive at 0.5 per cent. In the second group, only indigo blue (sodium sulphonate) was tested and found to approximate the last three in the limits set by inhibition. Sodium formate and indigo blue formerly found considerable favor in the cultivation of anaerobes in deep media (See Hammerl, 1901), but they are not now used at all; it is impossible to secure surface cultures by their aid alone.

Novy (1893) made a considerable study of glucose and other ingredients of culture media in relation to anaerobic cultures. He considered that glucose (1 to 2 per cent), gelatin (2 to 5 per cent) and litmus, all have valuable reducing properties and that an ideal medium should contain all three. The advantages of litmus were emphasized especially, since it, like indigo, combines

the properties of a reducing agent and an indicator, being decolorized in oxygen-free media.

So far, the successful use of chemical reducing agents had been restricted to organic compounds; the first inorganic substances to be found useful were sulfur compounds. Trenkmann (1898) determined the limits of concentration within which the bacilli of malignant oedema, tetanus and blackleg would grow in broth containing $Na_2S$ but the results are expressed in drops and therefore have no quantitative value. Hammerl (1901) compared the efficiency of $Na_2S$, $K_2S$ and $NH_4SH$ as reducing agents, judging the results not alone by the growth of anaerobic organisms, but also by the decolorization of methylene blue. $NH_4SH$ was found to be the most efficacious, from both standpoints. Its instability is a serious bar to its practical use, however. A further contribution was made by Rivas (1902) to the subject of cultivating anaerobes in the presence of $Na_2S$ and $NH_4SH$, the only tangible result of which was to show the futility of attempting to secure surface cultures by these means.

The reducing action of compounds containing sulfur has recently been studied anew by Hosoya (1925) and others. Hosoya found that the addition of 0.001 per cent l-cysteine hydrochloride greatly stimulated the growth of various obligately anaerobic bacteria in liquid media (pH 7.2 to 7.4) without seals other than depth and that neither l-cystine, taurine, nor thioglycollic acid could replace the l-cysteine for this purpose. In a further report, Hosoya and Kishino (1925) observed that *B. tetani* and *B. botulinus* failed to grow in a gelatin digest medium except when cysteine, sulfuretted hydrogen, cystine, or sodium sulfide ($Na_2S$) were added. Cysteine and sulfuretted hydrogen were the best and neither could be replaced by l-tryptophane, l-tyrosine, sodium sulfate ($Na_2SO_4$), acid sodium sulfate ($NaHSO_4$), sodium sulfite ($Na_2SO_3$), sodium thiosulfate ($Na_2S_2O_3$), taurine, or sodium taurocholate. The results with l-cysteine were duplicated for *B. sporogenes* by Quastel and Stephenson (1926) who attribute the effect to the reducing action of the SH group since thioglycollic acid (compare Hosoya) and glutathione behaved similarly. Quastel and Stephenson's findings with *B. sporogenes* were con-

firmed by Aubertin, Aubel, and Genevois (1928) and extended to *B. putrificus, B. fallax, B. perfringens* (*B. Welchii*), *B. bifermentans, B. oedematiens* (*B. Novyi*), and *B. tetani*. Interesting as they are, the results must be regarded for the present as primarily of only theoretical importance, since they have not made it possible to secure strictly aerobic, i.e. surface growth on solid media of any obligate anaerobe. Guillemard (1911) utilized iron sulfate (0.3 per cent) as a means of reduction. But the medium must be boiled out and growth cannot be secured on the surface of solid media. However, Guillemard found that six parts of iron per 100,000 profoundly affected the metabolism of certain anaerobic bacteria, and Scott (1926) observed that the addition of ferric sulfate to culture media aided *B. Chauvoei* to preserve its virulence. The reducing properties of iron and some of its compounds suggest that it may prove to have special values in anaerobic technic which still remain to be discovered.

Our knowledge of chemical reducing agents may be briefly related to certain biological theories of anaerobiosis which we have already reviewed in another place (Hall, 1928), for the ultimate explanation of aerobic-anaerobic symbiosis and the successful use of animal and plant tissues in the so called "aerobic culture" of obligate anaerobes, must rest in the last analysis upon a true understanding of their physical and chemical bases.

In 1922, M'Leod and Gordon (1922) discovered that the early death of the pneumococcus in cultures was apparently due to the accumulation of peroxide (of hydrogen?), for the production of which evidence was found in the green discoloration of "chocolate" blood agar, and in the liberation of oxygen from pneumococcus cultures by liver catalase. Streptococci and other bacteria also produce peroxide and it was suggested that the well known beneficial action of fresh tissues upon various bacteria is due to the destruction of detrimental peroxide by catalase.

That many bacteria also produce catalase has been known since the work of Gottstein (1893) and Beijerinck (1893). The latter also pointed out that the lactic acid bacteria do not produce it, and Lowenstein (1903) showed that the anaerobic bacteria resemble the lactic acid bacteria in this respect.

Callow (1923) suggested that, if the obligate anaerobes lack catalase, their inability to grow in the presence of oxygen might be explained by the toxic action of peroxide. In the absence of air, no peroxide would be formed and growth would occur. She showed that while nine aerobes produced abundant catalase, seven anaerobes and three streptococci produced only the slightest possible traces of this enzyme. Yet it was impossible to detect peroxide in anaerobic cultures exposed to the air; neither could the anaerobes be cultivated upon the surface of solid media containing catalase in contact with air. These results were confirmed by M'Leod and Gordon (1923) and supported by the information that *B. tetani*, *Vibrion septique*, and *B. Welchii* are highly susceptible to slight traces of added peroxide of hydrogen. As these investigators have suggested, their failure to demonstrate peroxide formation by anaerobic cultures exposed to the air does not necessarily invalidate their theory, since the amount of peroxide required to inhibit the growth of anaerobes is apparently much less than that necessary for its chemical demonstration. While they were unable to secure any evidenee that liver catalase favors the growth of *B. tetani*, good growth of *B. Welchii* was secured with catalase in oxygen tensions that tended to inhibit without it. They further found (1925) that there is a close parallelism between the reduction of glutathione, the dissolving of cystine, and the discoloration of chocolate blood agar and pointed out that all these phenomena can be explained by the assumption that the bacteria responsible for them generate active hydrogen.

Avery and Morgan (1923) (1924) were also able to cultivate certain obligately anaerobic bacteria in broth containing unheated plant tissues, for a few transfers. But surface cultures in air failed, as usual, even with *B. histolyticus*, which is really not an obligate anaerobe but can be cultivated aerobically for an apparently indefinite number of generations (Hall, 1923) (Hall and Whitehead, 1927). Perhaps the most important discovery of Avery and Morgan, was that the formation of peroxide by the pneumococcus is favored by free access of air and absence of catalase, while anaerobic conditions, or the presence of catalase

from plant tissues, inhibit the formation and accumulation of peroxide. But, like the English investigators, they were unable to demonstrate the formation of peroxides by the obligate anaerobes.

The first direct demonstration of peroxide formation by an obligate anaerobe was accomplished by Hagan (1924) with *Actinomyces necrophorus*, young cultures of which exposed to the air give the benzidine test for peroxide of hydrogen. But M'Leod and Gordon (1925) were unable to demonstrate peroxide in cultures of *B. tetani* and *B. Welchii* by Hagan's methods and the catalase-peroxide theory remains, for all obligate anaerobes except *A. necrophorus*, merely an interesting hypothesis supported only by indirect evidence. Yet Neill (1925) holds that the oxidation and reduction reactions of sterile plant tissues are essentially identical with those of pneumococci and anaerobic bacteria, and furnish the best explanation, thus far, for the beneficial action of *raw* plant and animal tissues upon anaerobic growth. On the other hand Novy (1925) holds that "the inability of anaerobes to grow in the air is not due to the hypothetical production of peroxide and to the absence of catalase" but "that the fundamental difference between obligative aerobes and anaerobes lies in the nature of the respiratory enzymes, which are designated as aerase and anaerase, respectively. The potato and the facultative anaerobes possess both types; that present in obligative anaerobes can function only in the absence of oxygen, while that of the aerobe can work only in the presence of oxygen." All living protoplasm respires and Novy's conception of the beneficial action of plant tissues upon anaerobic growth dispenses with the peroxide-catalase hypothesis and emphasizes the idea that obligate anaerobes grow in the presence of raw plant tissues because the latter reduce the oxygen tension, just as aerobic bacteria do, below the toxic concentration.

Useful as chemical reducing agents are in any culture medium for the growth of obligately anaerobic bacteria, we are forced to conclude that, as yet, none has been found that permits these organisms really to grow as aerobes grow, that is, upon the surface of a solid medium exposed to the atmosphere through a

cotton plug. As Hunziker said in 1902, "no matter how great the reducing power of any one of these chemical reducing agents may be, they are not able to make harmless the atmospheric oxygen, which re-enters the medium when the latter is poured into Petri dishes. . . . . In order to use reducing agents successfully, they should be used in connection with some other method for cultivating anaerobic bacteria."

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